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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Attorney Docket No. **PROV.02-02**
Date: April 9, 2002
Page 1 of 2

COMMISSIONER FOR PATENTS AND TRADEMARKS
WASHINGTON, D.C. 20231

ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37CFR §1.53(c).

For: A Multi-Modal Strategy for Effective Suppression of Diabetes

Inventors: **Habib Zaghouani**
Residence Address: 1608 Brookfield Manor; Columbia, MO 65203

Enclosed are:

(X) A Specification in 10 pages.

(X) Drawings in 8 pages.

(X) A Certificate of Mailing, Express Mail No. EJ499784466US.

(X) A Pre-paid Return Receipt Postcard.

(X) Please direct all correspondence to:
John Wurst, Patent Counsel
Alliance Pharmaceutical Corp.
3040 Science Park Road
San Diego, CA 92121

(X) Please charge Deposit Account No. 01-1008 in the amount of $80.00 for the Provisional Application Filing fee (Small Entity).

(X) If for some reason Applicant has not paid a sufficient fee for this application, please consider this an authorization to charge our Deposit Account No. 01-1008 for any fee, which may be due. Similarly, please credit any overpayment to Deposit Account No. 01-1008. A duplicate copy of this sheet is enclosed.

(X) If there are any questions concerning this application, please call the undersigned at the number stated below.

Respectfully submitted,

Dated: 4/9/02

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Commissioner for Patents and Trademarks
Washington, D.C. 20231

ATTN: PROVISIONAL PATENT APPLICATION

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Attorney Docket No.	:	PROV.02-02
Applicant	:	Zaghouani
For	:	A Multi-Modal Strategy for Effective Suppression of Diabetes
Attorney	:	JEW
"Express Mail" Mailing Label No.	:	EJ499784466US
Date of Deposit	:	April 9, 2002

I hereby certify that the accompanying

Provisional Application Transmittal in Duplicate; Specification in 10 pages; Drawings in 8 pages; and Return Prepaid Postcard

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and are addressed to the Commissioner for Patents and Trademarks, Washington, D.C. 20231

Christine Hughey

Christine Hughey

A Multi-Modal Strategy for Effective Suppression of Diabetes

Summary of the Invention

A number of strategies have been considered for the down-regulation of diabetogenic T cells and the modulation of the Th1-mediated autoimmune disease, insulin-dependent diabetes mellitus (IDDM). Recently, much focus has been directed towards regulatory T cells (Treg) and their suppressive function on pathogenic lymphocytes, as well as towards direct negative regulation of autoaggressive T cells. However, antigen driven approaches for the stimulation of Treg cells for specific suppression of autoimmunity in type 1 diabetes have yet to be defined.

The present application demonstrates a strategy that suppresses autoimmunity associated with diabetes. Accordingly, the insulin β chain (INSβ) peptide 9-23 is genetically grafted onto an immunoglobulin (Ig) and the resulting Ig-INSβ chimera bound to the Fc receptor on APCs, which facilitated both peptide loading onto MHC molecules and suppression of autoimmune process. Furthermore, treatment of non-obese diabetic ("NOD") mice with aggregated Ig-INSβ at insulitis onset is shown to protect against development of diabetes. Knowing that IL-10 secreted by the APCs during presentation of aggregated Ig-INSβ suppresses IFNγ production by T cells, it is believed that prevention of IDDM by aggregated Ig-INSβ operates through the down-regulation of previously activated Th1 cells and the induction of Treg cells that sustain such suppression. Thus, the multi-modal system described in the present application may be a successful strategy for the effective prevention of diabetes.

Brief Description of the Drawings:

Figure 1 shows results indicating that INSβ and HEL nucleotide sequences were successfully inserted within the CDR3 of the 91A3V_H gene;

Figure 2 shows results demonstrating the pairing of the 91A3H chain expressing INSβ peptide with the parental 91A3L chain;

Figures 3A - 3D indicate that Ig-INSβ chimera were presented to specific T cells;

Figures 4A - 4C shows correlation of serum anti-insulin antibody with the development of diabetes;

Figures 5A and 5B show results demonstrating dose dependent suppression of diabetes by soluble (sol) Ig-INSβ in IAA-positive NOD mice;

Figure 6 shows results demonstrating that soluble Ig-INSβ slightly delays diabetes when administered at pre-insulitis stage;

Figures 7A and 7B shows results demonstrating that Agg Ig-INSβ slightly delays diabetes in 1-AA positive mice; and,

Figure 8 shows that Agg Ig-INSβ delays diabetes when administered at the insulitis stage.

Detailed Description of the Invention

Expression of INSβ and HEL peptides on Ig molecules drives efficient presentation to T cells.

Recent studies revealed that SJL mice with an ongoing experimental allergic encephalomyelitis (EAE) (EAE is a murine autoimmune disorder which is used as a model of multiple sclerosis in humans) recover from disease when treated with an aggregated Ig expressing the proteolipid protein-1 (PLP1) sequence 139-151 . The present application demonstrates that delivery of a diabetogenic peptide on Igs inhibits diabetes in the the non-

obese diabetic mouse ["NOD mouse"]. The NOD mouse is used as a model for type 1 diabetes in humans, which is an autoimmune disease with different mechanisms compared to multiple sclerosis, mediated by T cell reactive to islet-associated antigens.

The I-A^{g7}-restricted INSβ peptide, defined to be associated with the development of diabetes in the NOD mouse, was selected for expression on Igs and suppression of diabetes. HEL peptide, which is also presented by I-A^{g7} MHC class II molecules without causing diabetes, was used to generate an Ig-HEL chimera to serve as a control. Accordingly, INSβ and HEL nucleotide sequences were separately inserted into the CDR3 of the 91A3 heavy chain by PCR mutagenesis and the resulting chimeric heavy chain genes were analyzed by DNA sequencing. Comparison of the nucleotide sequence of the parental $91A_3V_H$ gene to the sequence of the chimeric $91A_3V_H$-INSβ indicates that the full INSβ sequence was inserted in the correct reading frame in place of the D segment. Similar comparison indicate that HEL sequence was fully inserted within the CDR3 region in place of the D segment also in the correct reading frame.

Figure 1 shows the nucleotide sequences of these inserts as well as the flanking regions surrounding them. The results in Figure 1 indicate that INSβ nucleotide sequence was fully inserted in place of the D segment. The flanking regions surrounding INSβ are identical to those regions flanking the D segment within the parental heavy chain indicating that the INSβ nucleotide sequence was inserted in the correct reading frame. Similarly, results were obtained with HEL peptide indicating that a full nucleotide sequence of HEL peptide was incorporated in the correct reading frame (see Figure 1).

Subsequently, these chimeric heavy chain genes were subcloned into a pSV2 expression vector and separately co-transfected with the parental kappa light chain gene into the non-Ig-secreting myeloma B cell line SP2/0 as follows: the $91A3V_H$-INSβ fragment was subcloned into

an expression vector in front of the exons coding for the constant region of a BALB/c γ2b. This plasmid was then co-transfected into the non-Ig-producing SP2/0 myeloma B cell line with an expression vector carrying the parental 91A3 light chain. Transfectants producing Ig-INSβ were selected in the presence of geneticin and mycophenolic acid. Detection of complete Ig-INSβ in the supernatant from transfectoma cells was carried out by incubation of supernatant of Ig-INSβ or Ig-W transfectants on microtiter plates coated with rabbit anti-mouse γ2b -chain specific antibody and revelation of captured Ig-chimeras with [^{125}I]-labeled rat anti-mouse kappa light chain mAb. Each bar represents the mean ± SD of triplicates.

Using selective drugs, wells with cell growth were identified visually, and the supernatant was tested for presence of Igs. As depicted in Figure 2, supernatant from a representative Ig-INSβ transfectant incubated on plates coated with anti-γ2b antibody bound a rat anti-mouse kappa light chain mAb, as did Ig-W, the parental 91A3 antibody with an intact CDR3 domain indicating that the 91A3-INSβ chimeric heavy chain paired with the parental light chain and formed a complete Ig-INSβ molecule. Similarly, a representative supernatant from a 91A3-HEL transfectant showed significant binding of the anti-light chain antibody indicating that insertion of the HEL peptide within the heavy chain variable region did not alter pairing with the parental light chain and a complete Ig-HEL molecule was produced.

The next question was whether Ig-INSβ is processed properly and generates an INSβ peptide that could be presented to T cells. To test this premise, the chimera was purified from the supernatant of large-scale cultures by affinity chromatography and assayed for presentation using an INSβ-specific T cell clone that has been generated in the NOD mice by immunization with INSβ peptide. Similarly, to insure that HEL peptide could be processed from Ig-HEL and presented to T cells, an HEL-specific hybridoma was generated by fusing HEL-specific short-

term T cell line with the αβ-T cell receptor (αβ-TCR)-negative thymoma BW1100. As described in greater detail, irradiated (3000 rads) NOD splenocytes (5 x 10^5 cells/50μl/well) were incubated with 100 μl antigen and one hour later T cells (5 x 10^4 cells/well/ 50 μl) specific for either INSβ (a and b) or HEL (c and d) peptide were added. For presentation of INSβ peptide (a) and Ig-INSβ (b), since the T cells were from a line, the activation was assessed by [^{3}H]thymidine incorporation. Accordingly, 1 μCi [^{3}H]thymidine per well was added during the last 14 hours of a 3-day incubation period and the cells were harvested, and the radioactivity counted. For presentation of HEL peptide (c) and Ig-HEL (d), the T cell activation was assessed by measuring IL-2 production as the HEL-specific cells were from a hybridoma. Accordingly, after 24 hour incubation, IL-2 was measured in 100 μl supernatant by ELISA. In this assay, the peptides were used at 10 μM concentration and the Ig-chimeras at 1 μM. Each point or bar represents the mean of triplicates.

As indicated in Figures 3a - 3d, the INSβ-specific T cell clone proliferated significantly upon incubation with irradiated NOD splenic APCs and INSβ peptide (Figure 3a), or Ig-INSβ (Figure 3b) indicating that Ig-INSβ is taken up by the APCs and an INSβ peptide is generated and presented to T cells. HEL peptide and Ig-HEL stimulate the HEL-specific hybridoma as measured by IL-2 production (Figures 3c and 3d), but were unable to induce proliferation of the INSβ specific line. This indicates that the presentation of INSβ and Ig-INSβ is specific.

Insulin-specific autoantibodies (IAA) can serve as a marker for early development of diabetes.

Gender study of the incidence of diabetes in our NOD colony indicated that 38% of male NOD mice develop spontaneous diabetes by the age of 25 weeks. However, female NOD mice have shown a greater susceptibility and 85% developed spontaneous diabetes at week 25 of age.

This is in good agreement with previous reports and suggest that the use of female mice would be more suitable for our investigation. Recently, it has been shown that insulin autoantibodies (IAA) can be used as a marker for prediction of type I diabetes in children and young NOD mice. This is advantageous as it facilitates early intervention for suppression of the disease without compromising the accuracy of the study, and circumvents the use of animals that are unlikely to develop diabetes. Therefore, we have opted to develop a chart to include only IAA-positive mice to assess the ability of Ig-INSβ for suppression of diabetes. Accordingly, a group of 70 NOD female mice was subject to weekly testing for IAA beginning at week 6 through week 12 of age and the IAA-positive mice were monitored for blood glucose thereafter and up to 30 weeks. In Fig. 4(a), 70 adult female NOD mice were bled weekly starting at the age of 6 weeks and their serum samples were tested for insulin autoantibodies (IAA) at a 1/200 dilution by ELISA. A sample is considered IAA positive when the OD_{405} is > 0.2. This cutoff line of 0.2 was chosen because serum samples from 10 SJL mice which are non-prone to diabetes and presumably do not produce insulin specific autoantibodies never exceeded 0.2 OD_{405}. Among the 70 mice tested, 58 (83%) have shown an IAA positive test. In Fig. 4(b), the 58 mice that tested IAA-positive by week 12 were subjected to weekly measurement of blood glucose beginning at week 12 through week 30. A mouse is considered diabetic when the blood sugar is 300 mg/dl or above for two consecutive weeks. Among the 58 IAA-positive mice, 49 (84%) became diabetic by week 30 of age. Fig. 4(c) shows the percent incidence of early (15-20 weeks of age) and delayed (21 to 30 weeks of age) diabetes for mice developing IAA at the indicated weeks. (n, indicates the number of mice per group).

As indicated in Figures 4(a) - 4(c), the appearance of IAA begins at week 7. By 12 weeks of age, 58 among the 70 mice tested (83%) had become IAA-positive. Furthermore, among the

58 IAA-positive mice, 84% had become diabetic by 30 weeks of age (Figure 4b) indicating that IAA can serve as a marker for the development of type I diabetes. Interestingly, a significant percentage (60%) of the mice that become IAA-positive at week 8, 9, or 10 manifested diabetes at the age of 15 to 20 weeks and such early incidence rose to 80% for the mice which developed IAA at week 11 of age. Therefore, these results suggest that development of IAA between the age of 8 to 11 weeks can serve as a marker for the development of diabetes at the early age of 15 to 20 weeks.

Soluble Ig-INSβ slightly delays diabetes when administered at the early stage of insulitis or subsequent to the development of IAA antibodies.

IAA-positive mice that seroconverted displaying IAA at the age of 8 to 11 weeks, were then given twice sol Ig-INSβ in saline on the week of seroconversion and 7 days later and were monitored for blood glucose up to week 30 of age. Groups of female NOD mice (10 per group) that were diagnosed positive for IAA between the age of 7 and 11 weeks were given an intraperitoneal injection of 100 (a) or 200 (b) μg of either sol Ig-INSβ (black bars) or sol Ig-HEL (gray bars) on the week of seroconversion and 7 days after. A fifth group did not receive any injection (open bars: Nil) and was incorporated in both panels to serve as control. Shown is the percentage of incidence of diabetes in each of the five groups at week 16, 20, 26 and 30. As can be seen in Figure 5, two doses of 100 μg Ig-INSβ had no significant impact on diabetes while two doses of 200 μg Ig-INSβ delayed diabetes up to week 16. At week 20 of age, while 50% of the untreated mice became diabetic, only 30 % of those given Ig-INSβ developed diabetes. By week 26 most of the Ig-INSβ animals developed diabetes. The delay of diabetes is antigen specific as Ig-HEL had no significant protection against diabetes (Figures 5a - 5b).

Ig-INSβ was then tested for protection against diabetes before IAA-seroconversion. Accordingly, NOD mice were given 300 μg Ig-INSβ on week 4, 5, and 6 of age and monitored for blood glucose weekly up to week 30 of age. Groups of female NOD mice (10 per group) were given an intraperitoneal injection of a saline solution containing 300 μg of either sol Ig-INSβ (black bars) or Ig-HEL (gray bars) at week 4, 5, and 6 of age. A third group that did not receive any injection (open bars:Nil) was included for control purpose. Shown is the percentage of incidence of diabetes in each of the 3 groups at week 16, 20, 26 and 30. The results presented in Figure 6 indicate that while 30% of untreated mice developed diabetes by week 16 of age none of the mice treated with Ig-INSβ became diabetic at that age. No delay was observed in the mice treated with the control Ig-HEL indicating that the protection against diabetes is antigen specific. Furthermore, by week 20, while 50% of the untreated or Ig-HEL treated mice became diabetic, only 30% of the Ig-INSβ group advanced to diabetes. At the age of 30 weeks, while 8 mice in each of the untreated of Ig-HEL treated groups became diabetic, only 6 mice of the Ig-INSβ progressed to developing the disease.

Aggregated Ig-INSβ effectively delays the development of diabetes when administered at the pre-insulitis stage.

Aggregation of Igs confers Fc associated functions such as cross-linking of FcRs and activation of complement. It has been previously shown that aggregation of Ig-myelin chimeras derived from the same backbone as Ig-INSβ and Ig-HEL and therefore using the IgG2b isotype, cross-link FcγRs on APCs and induce the production of IL-10 by dendritic cells and macrophages. In addition, Ig-PLP1, a chimera carrying PLP1 peptide corresponding to amino acid residues 139-151 of proteolipid protein (PLP), induced a slight recovery from experimental

allergic encephalomyelitis (EAE) when it was administered into diseased mice in a soluble form. However, aggregated Ig-PLP1 induced full recovery from EAE and neutralization of endogenous IL-10 by co-administration of anti-IL-10 antibody restored disease severity. These results indicated that cross-linking of FcγRs and IL-10 production by APCs potentiate the modulatory function of Ig-PLP1 and promote effective suppression of disease. To test whether similar effects could be achieved in the NOD model, Ig-INSβ was aggregated and assayed both at the insulitis stage and after seroconversion for protection against diabetes. Groups of female NOD mice (10 per group) that were diagnosed positive for IAA between the age of 7 and 11 weeks were given an intraperitoneal injection 100 (a) or 200 (b) μg of either agg Ig-INSβ (black bars) or agg Ig-HEL (gray bars) on the week of seroconversion and 7 days after. A fifth group did not receive any injection (open bars:Nil) and was incorporated in both panels to serve as control. Shown is the percentage of incidence of diabetes in each of the five groups at week 16, 20, 26 and 30 of age.

As can be seen in Figures 7a - 7b, in IAA-positive mice, treatment with aggregated Ig-INSβ had no significant protective effect against diabetes when Ig-INSβ was given at a dose of 100 μg on the week of seroconversion and 7 days later. When the dose was increased to 200 μg per injection, some delay of the development of diabetes to week 20 took place: in the untreated group, 50% of the mice developed diabetes while in the Ig-INSβ, only 30% had diabetes. However, by week 26, there was as many diabetic mice in the Ig-HEL treated mice as in the Ig-INSβ mice.

In another experiment, groups of female NOD mice (10 per group) were given an intraperitoneal injection of a saline solution containing 300 μg of either agg Ig-INSβ (black bars) or agg Ig-HEL (gray bars) at week 4, 5, and 6 of age. A third group that did not receive

any injection (open bars:Nil) was included for control purpose. Shown is the percentage of incidence of diabetes in each of the 3 groups at week 16, 20, 26 and 30 of age. In contrast to the results shown in Figures 7a - 7b, when Ig-INSβ was given at the pre-insulitis stage, complete protection was observed at week 16 and at week 20. While five mice (50%) developed diabetes in the untreated group, only 1 mouse (10%) had diabetes in the Ig-INSβ group. Furthermore, at week 26, the protection against diabetes was still significant as 8 out 10 mice (80%) became diabetic in the untreated group, while only 3 out of 10 (30%) had the disease in the Ig-INSβ treated group (Figure 8). Ig-HEL treatment has protective effect as none of the mice treated with Ig-HEL developed diabetes by week 16 and the number of mice with diabetes at week 20 and 26 was still below the number of diabetic mice in the untreated group. This effect maybe due to bystander effect of IL-10 induced by the aggregation of Ig-HEL or displacement of agonistic disease associated epitopes from I-Ag^7 molecules.

Figure 1

$91A_3H$ ——— D ———

TYR PHE CYS ALA ARG SER TYR TYR SER GLY ASP MET TYR CYS PHE ASP TYR TRP

TAT TTC TGT GCA AGA TCG TAT TAC TCT GGT GAT ATG TAC TGC TTT GAC TAC TGG

$91A_3H$-INSβ ——— INSβ ———

\- - - - - - HIS LEU VAL GLU ALA LEU - LEU VAL CYS GLY GLU ARG GLY - - - -

--- --- --- --- --- AGC C-C CTA GTG -AG -CG C-T --- CT- GTT TGC GGT GAA AGA GGT --- --- --- ---

$91A_3H$-HEL ——— HEL ———

\- - - - - ALA MET LYS ARG HIS GLY LEU ASP ASN TYR ARG GLY TYR SER LEU - - - -

--- --- --- --- --- GCA ATG A-G CGC CAC -GG A-A G-- AA- TAT CGG GGA TAT AGC CTC --- --- --- ---

Figure 2

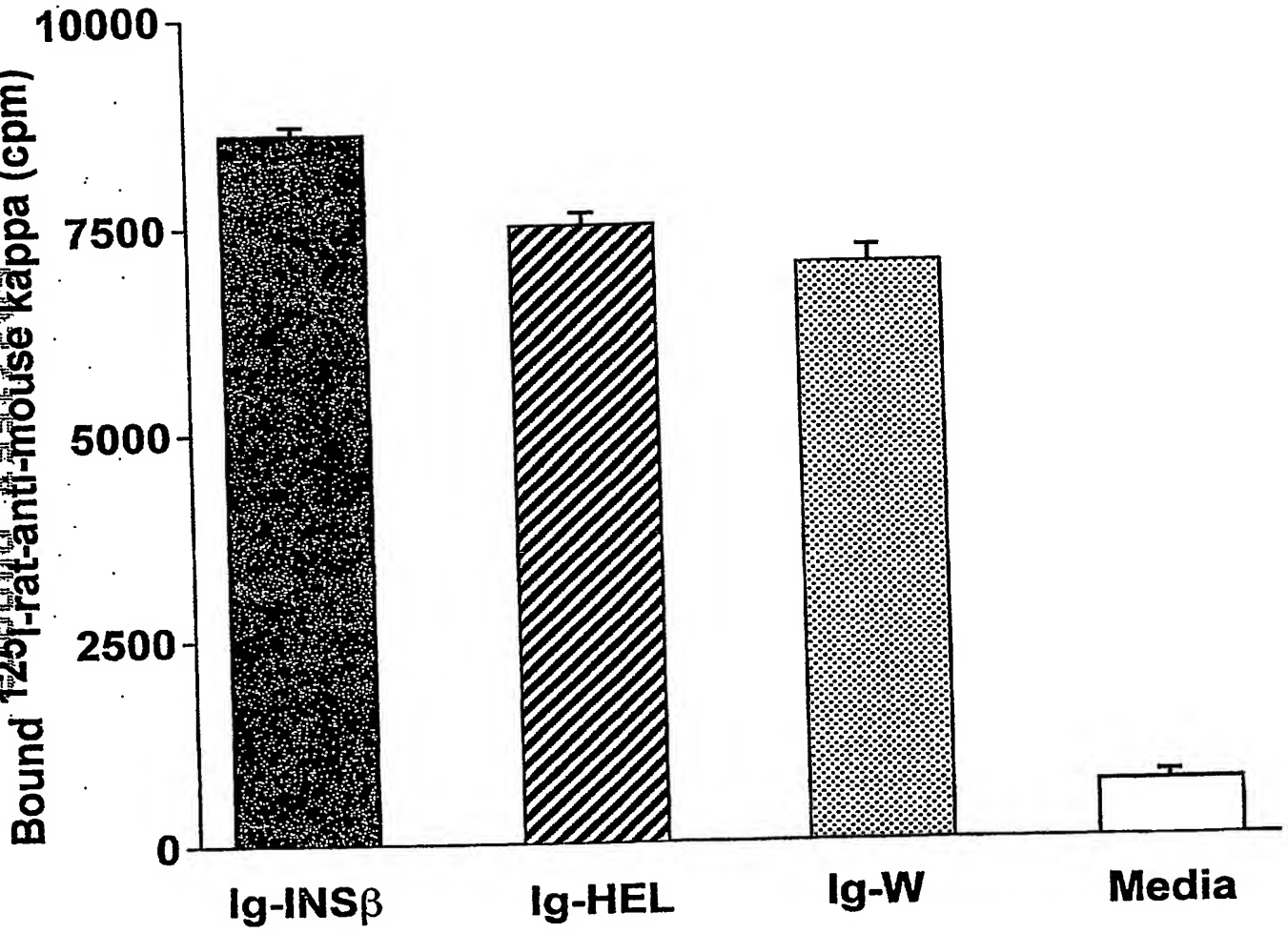
10000
7500
5000
2500
0
Bound 125I-rat-anti-mouse kappa (cpm)
Ig-INSβ
Ig-HEL
Ig-W
Media

Figure 3

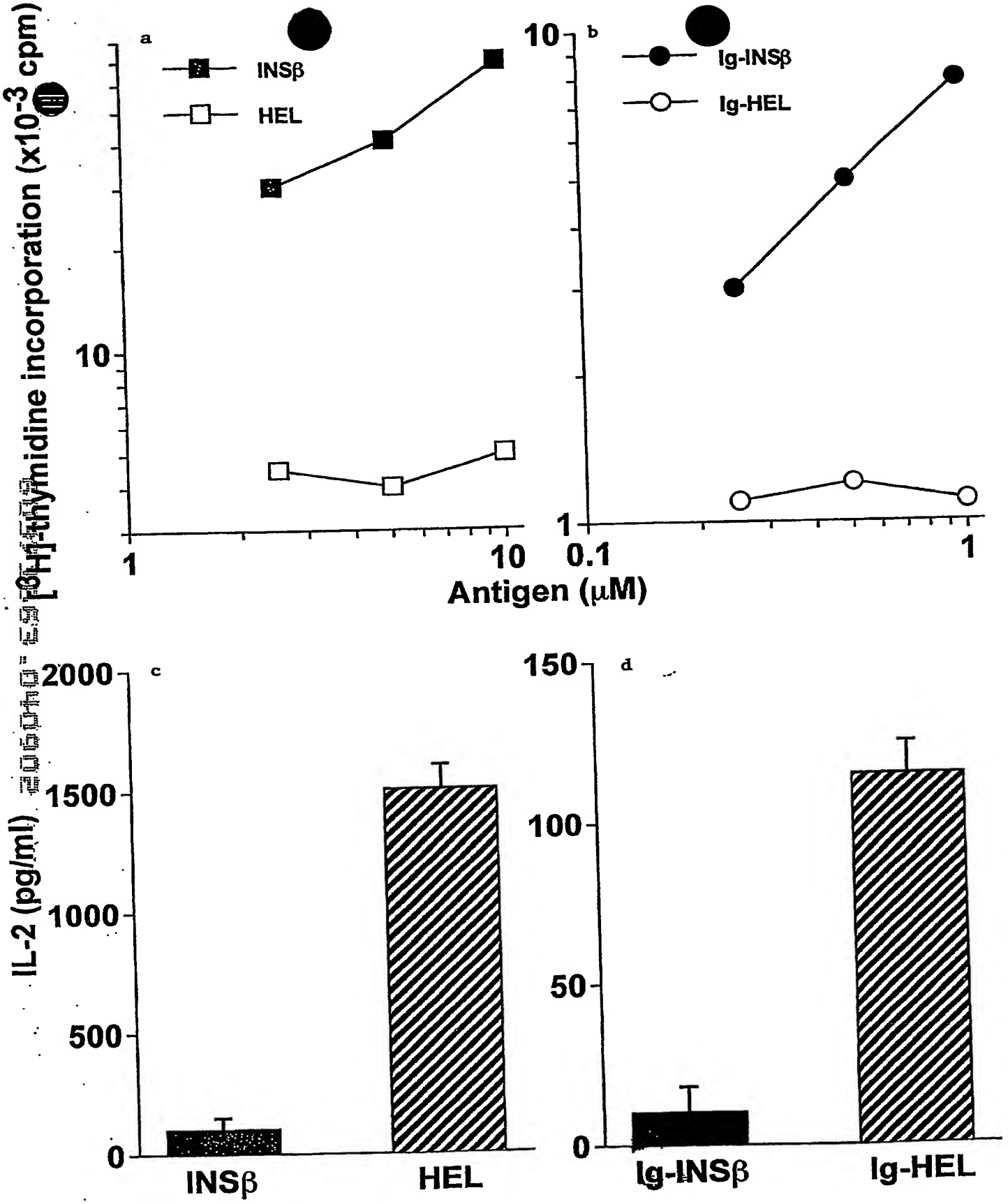
a
INSβ
HEL
10
1
10
b
Ig-INSβ
Ig-HEL
10
1
0.1
1
Antigen (μM)
[3H]-thymidine incorporation (x10-3 cpm)
c
2000
1500
1000
500
0
INSβ
HEL
d
150
100
50
0
Ig-INSβ
Ig-HEL
IL-2 (pg/ml)

Figure 4

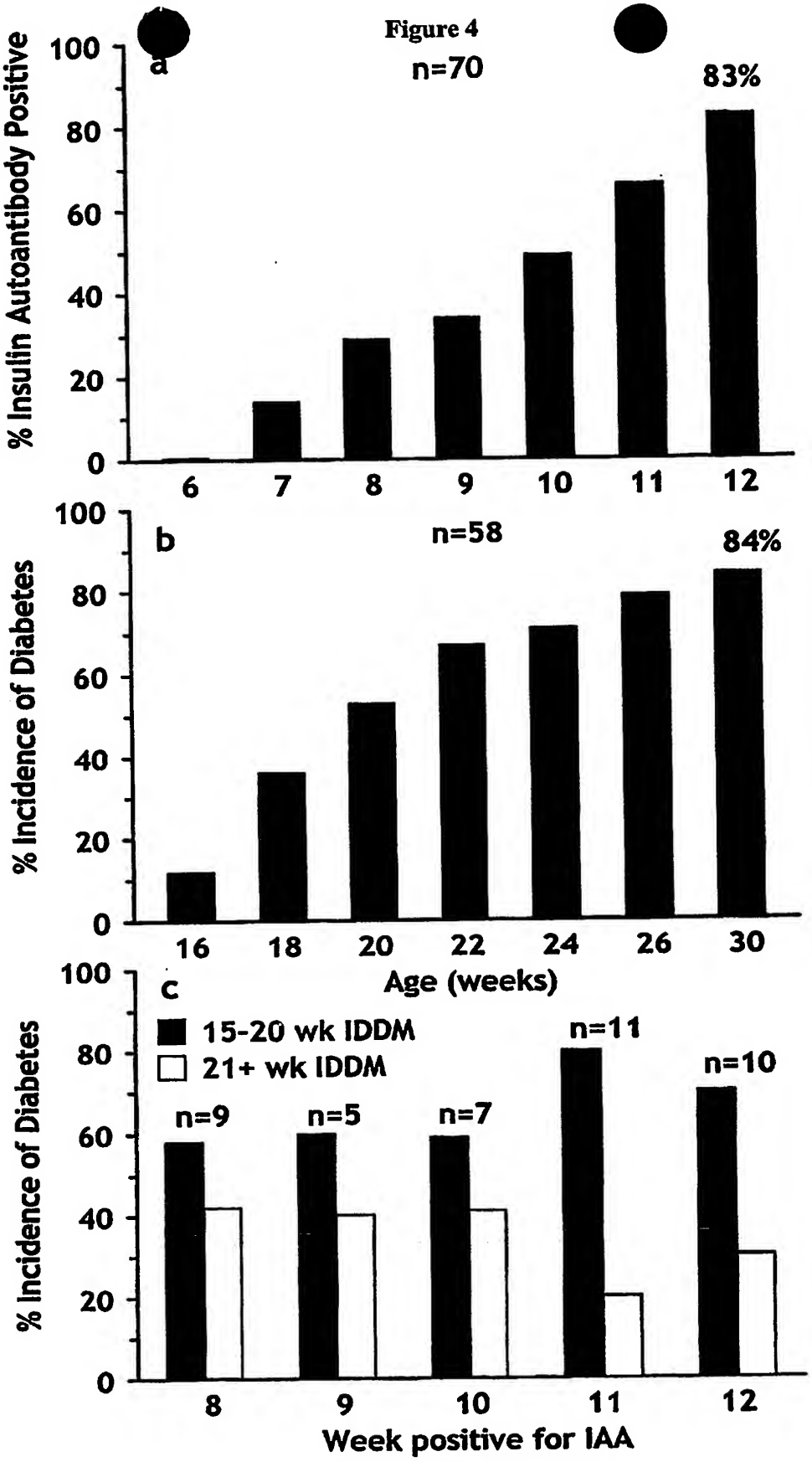
a
n=70
83%
% Insulin Autoantibody Positive
100
80
60
40
20
0
6
7
8
9
10
11
12
b
n=58
84%
% Incidence of Diabetes
16
18
20
22
24
26
30
Age (weeks)
c
15-20 wk IDDM
21+ wk IDDM
n=9
n=5
n=7
n=11
n=10
% Incidence of Diabetes
8
9
10
11
12
Week positive for IAA

Figure 5

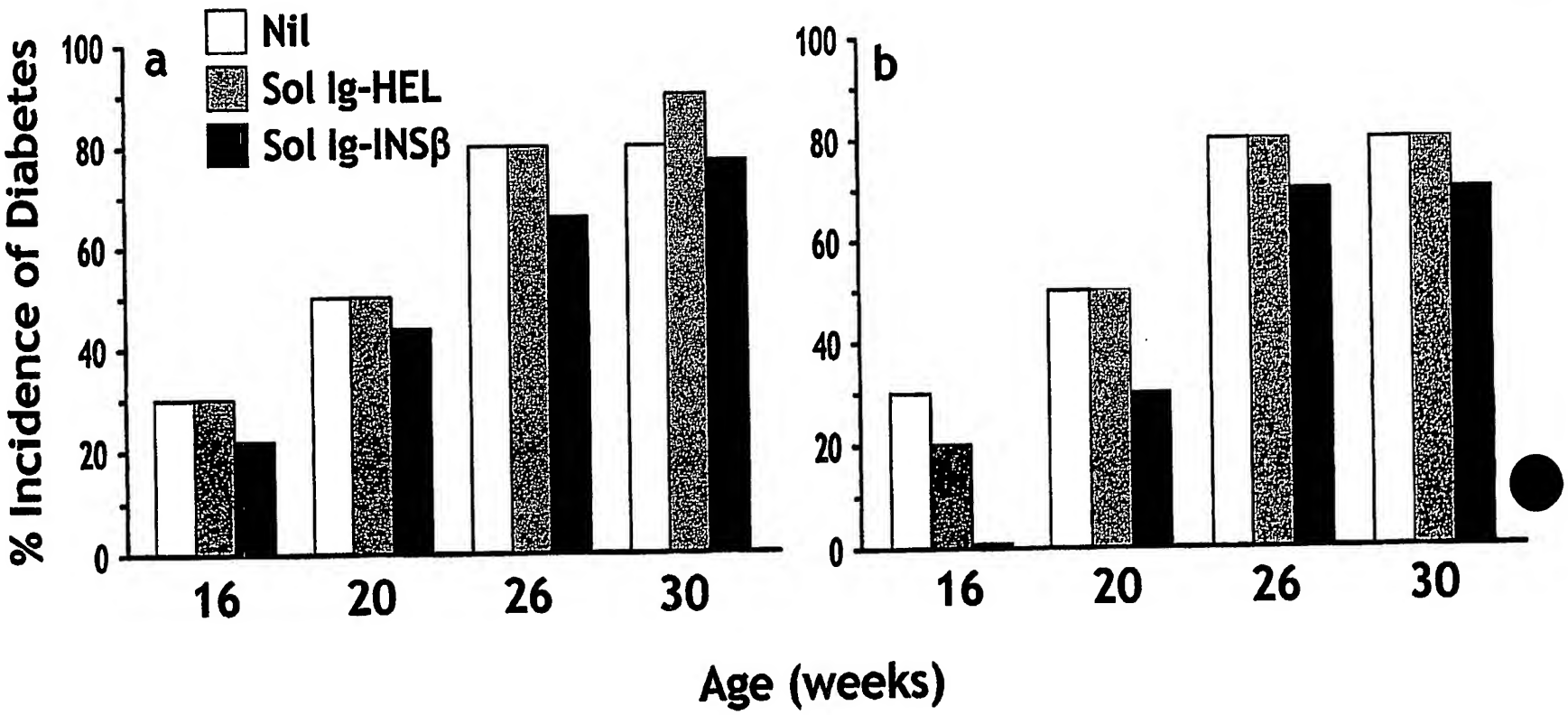

Figure 6

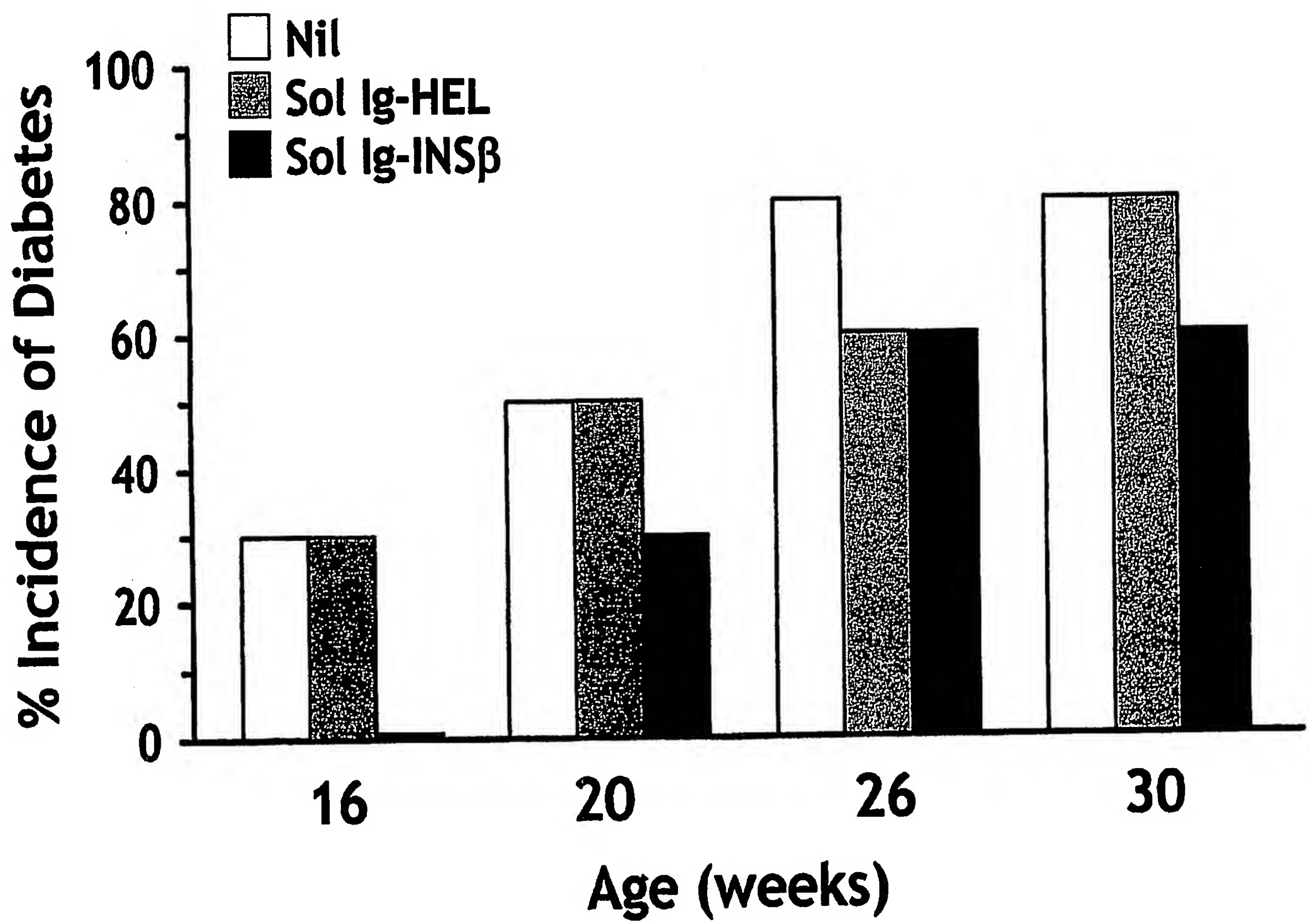
Nil
Sol Ig-HEL
Sol Ig-INSβ
% Incidence of Diabetes
100
80
60
40
20
0
16
20
26
30
Age (weeks)

Figure 7

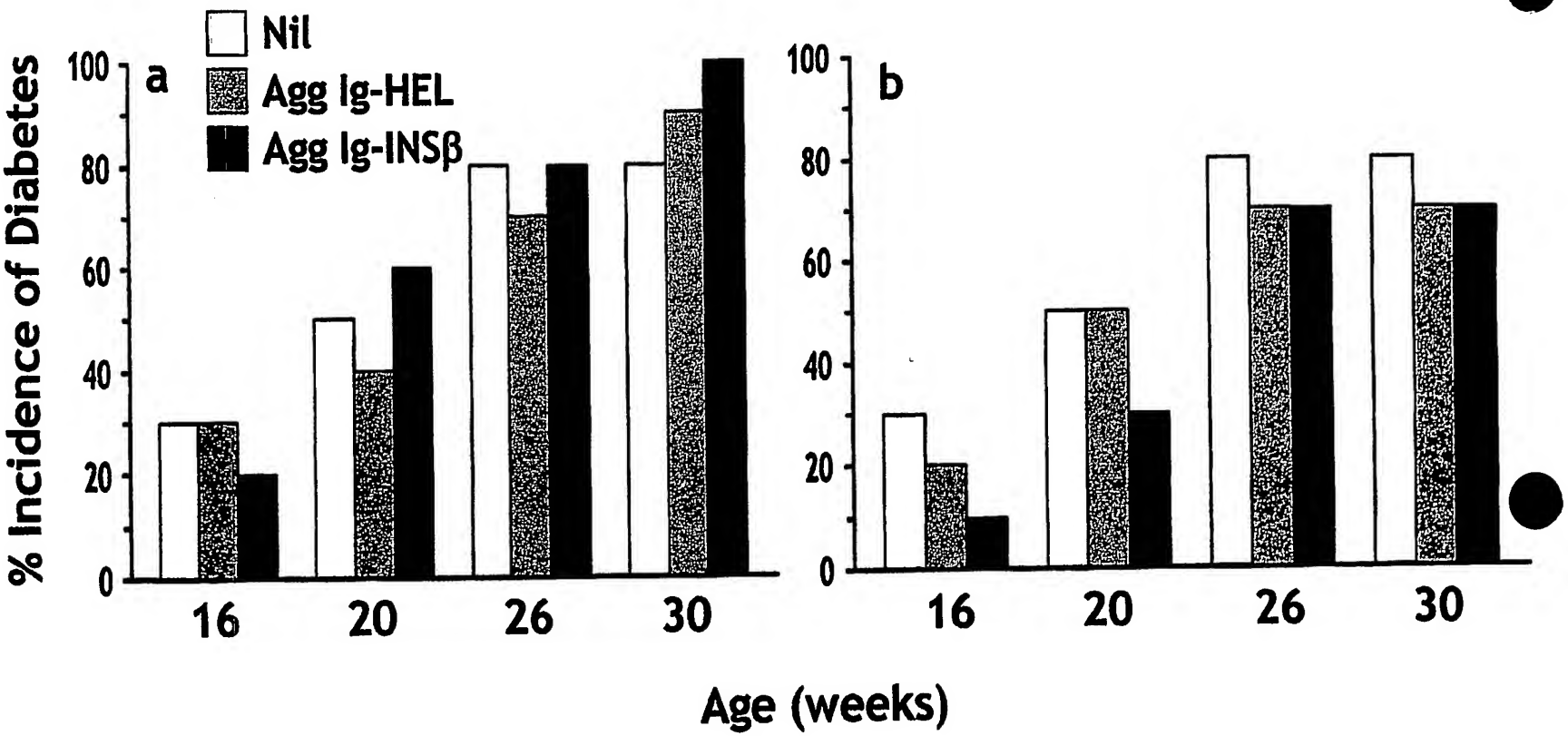
Nil
Agg Ig-HEL
Agg Ig-INSβ
a
b
% Incidence of Diabetes
100
80
60
40
20
0
16
20
26
30
Age (weeks)

Figure 8